Institute Report No. 244

Genotoxic Potential of Reactive-Polymer in the CHO HGPRT Forward Mutation Assay and CHO Sister Chromatid Exchange Assay

John W. Harbell, PhD, MAJ MSC
Lillie D. Witcher, BS, SGT USA
and
Don W. Korte, Jr., PhD, MAJ MSC

GENETIC AND CELLULAR TOXICOLOGY BRANCH
DIVISION OF TOXICOLOGY

AUGUST 1987

Toxicology Series 221

LETTERMAN ARMY INSTITUTE OF RESEARCH
PRESIDIO OF SAN FRANCISCO, CALIFORNIA 94129

Genetic Potential of Reactive-Polymer in the CHO HGPRT Forward Mutation Assay and CHO Sister Chromatid Exchange Assay (Harbell, Witcher, and Korte)

This material has been reviewed by Letterman Army Institute of Research and there is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author(s) and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense *(AR 360-5)*

Edwin S Beatrice 12 Aug 87

Edwin S Beatrice (date)
COL, MC
Commanding

63775A

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No 0704-0188 Exp Date Jun 30 1986

AD-A190 516

1a REPORT SECURITY CLASSIFICATION: Unclassified	1b RESTRICTIVE MARKINGS
2a SECURITY CLASSIFICATION AUTHORITY	3 DISTRIBUTION/AVAILABILITY OF REPORT: Unlimited
2b DECLASSIFICATION/DOWNGRADING SCHEDULE	
4 PERFORMING ORGANIZATION REPORT NUMBER(S): Institute Report No. 244	5 MONITORING ORGANIZATION REPORT NUMBER(S)
6a NAME OF PERFORMING ORGANIZATION: Division of Toxicology; 6b OFFICE SYMBOL (If applicable): SGRD-UL-TO	7a NAME OF MONITORING ORGANIZATION: LAIR
6c ADDRESS (City, State, and ZIP Code): Letterman Army Institute of Research San Francisco, Ca 94129-6800	7b ADDRESS (City, State, and ZIP Code): San Francisco, CA 94129-6800
8a NAME OF FUNDING/SPONSORING ORGANIZATION: USAMRDC; 8b OFFICE SYMBOL (If applicable)	9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
8c ADDRESS (City, State, and ZIP Code): Fort Detrick, MD 21701-5012	10 SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO 3M16277A875; PROJECT NO WU 306; TASK NO; WORK UNIT ACCESSION NO TLE0

11 TITLE (Include Security Classification)
Genotoxic Potential of Reactive-Polymer in the CHO HGPRT Forward Mutation Assay and the CHO Sister Chromatid Exchange Assay

12 PERSONAL AUTHOR(S)
John W Harbell, MAJ, MS, Lillie D.Witcher, SGT, USA, and Don W. Korte Jr. MAJ, MS

13a TYPE OF REPORT	13b TIME COVERED	14 DATE OF REPORT (Year, Month, Day)	15 PAGE COUNT
Final	FROM _____ TO _____	August 1987	8

16 SUPPLEMENTARY NOTATION

17 COSATI CODES			18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Mutagenicity, Genetic Toxicology, HGPRT, Sister chromatid exchange, Chinese Hamster Ovary Cells, Reactive-polymer

19 ABSTRACT (Continue on reverse if necessary and identify by block number)

Reactive-polymer and its component parts were tested for their abilities to induce forward mutations in the CHO HGPRT Forward Mutation Assay and to damage DNA as measured by the CHO Sister Chromatid Exchange Assay All materials were tested over dose ranges to include cytotoxic and noncytotoxic doses. Neither reactive-polymer nor its components induced a significant rise in the mutant frequency in the CHO HGPRT assay or in the frequency of sister chromatid exchanges. These data suggest a low genotoxic potential for reactive-polymer.

20 DISTRIBUTION/AVAILABILITY OF ABSTRACT	21 ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	Unclassified
22a NAME OF RESPONSIBLE INDIVIDUAL: Edwin S. Beatrice	22b TELEPHONE (Include Area Code): 415-561-3600; 22c OFFICE SYMBOL: SGRD-UL-Z

DD FORM 1473, 84 MAR 83 APR edition may be used until exhausted
All other editions are obsolete

SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

ABSTRACT

Reactive-polymer and its component parts were tested for their abilities to induce forward mutations in the CHO HGPRT Forward Mutation Assay and to damage DNA as measured by the CHO Sister Chromatid Exchange Assay. All materials were tested over dose ranges to include cytotoxic and noncytotoxic doses. Neither reactive-polymer nor its components induced a significant rise in the mutant frequency in the CHO HGPRT assay or in the frequency of sister chromatid exchanges. These data suggest a low genotoxic potential for reactive-polymer.

Key Words: Mutagenicity, Genetic Toxicology, HGPRT, Sister chromatid exchange, Chinese Hamster Ovary Cells, Reactive-polymer

PREFACE

TYPE REPORT: Genetic Toxicology Study Report

TESTING FACILITY: Letterman Army Institute of Research
Presidio of San Francisco, CA 94129-6800

SPONSOR: US Army Medical Research and Development Command
Letterman Army Institute of Research
Presidio of San Francisco, CA 94129-6800

PROJECT/WORK UNIT/APC: Toxicity Testing of Antidotes for Chemical Warfare Agents; Work Unit: 360; APC: TLEO

STUDY NUMBER: 74028

STUDY DIRECTOR: MAJ Don W. Korte Jr., PhD, MSC

PRINCIPAL INVESTIGATOR: MAJ John W. Harbell, PhD, MSC

TEST SUBSTANCE: Reactive-polymer (TP072)

INCLUSIVE STUDY DATES: 29 Oct 86 to 8 May 87

OBJECTIVE: The objective of this study was to determine the genotoxic potential of reactive-polymer (TP072) by using the CHO HGPRT Forward Mutation Assay and the CHO Sister Chromatid Exchange Assay.

ACKNOWLEDGMENTS

The authors wish to thank Sp4 Suzanne E. Sebastian for technical assistance.

SIGNATURES OF PRINCIPAL SCIENTISTS AND MANAGERS INVOLVED IN THE STUDY

We, the undersigned, declare that this study number 74028 was performed under our supervision, according to the procedures described herein, and that this report is an accurate record of the results obtained.

30 July 87

JOHN W. HARBELL, PhD / Date
MAJ, MS
Principal Investigator

Lillie D. Witcher 30 July 87

LILLIE D. WITCHER B.S./ Date
SGT, USA
Co-Principal Investigator

30 July 87

DON W. KORTE, JR PHD / Date
MAJ, MS
Study Director

TABLE OF CONTENTS

The Genotoxic Potential of Reactive-polymer in the CHO HGPRT Forward Mutation Assay and the CHO Sister Chromatid Exchange Assay -- Harbell, Witcher, and Korte

Reactive-polymer is a potential reactive barrier against chemical agents. The Division of Toxicology, LAIR, was tasked by U.S. Army Natick Research, Development, and Engineering Center to assess the genotoxic potential of this material. Two distinct genotoxicity end points were chosen for this study: forward mutation and DNA damage. The first was assessed using the Chinese Hamster Ovary (CHO) hypoxanthine-guanine phosphoribosyl transferase (HGPRT) forward mutation assay (1) while the second was tested using the CHO sister chromatid exchange assay (2). These assays are normally conducted in this laboratory by following the Good Laboratory Practices (GLP) guidelines promulgated by the EPA. Due to the security classification of the material being tested, the assays for chemical composition and the independent audit required by these guidelines could not be employed. However, with the two exceptions listed above, the studies were conducted in accordance with GLP guidelines.

Objective of the Study

To examine the cytotoxicity, mutagenicity, and DNA-damaging capacity of reactive-polymer by using the CHO HGPRT forward mutation and CHO sister chromatid exchange assays.

MATERIALS AND METHODS

Test Compound

Reactive-polymer, its active components, and matrix components were evaluated in these assays. Reactive-polymer and its matrix were received as granules while the active components were a powder. The granules were ground to a fine powder by using a Spex liquid nitrogen mill (Spex Industries, Edison, New Jersey), sterilized in 70% ethanol for 20 minutes and then washed three times with sterile glass distilled water. All other materials were dissolved in sterile glass-distilled water and filter-sterilized. All dosing materials were prepared immediately before use.

Positive Control Chemicals

Ethyl methanesulfonate (EMS) (Sigma lot# 83F-0279) was dissolved in sterile glass-distilled water, filter-sterilized

through a 0.22 um filter and added to the culture medium to achieve the desired final concentrations.

Cells

CHO cells subline CHO-K_1-BH_4 used for the HGPRT assay were obtained from Dr. A. Hsie, Oak Ridge National Laboratory, Oak Ridge, Tennessee. These cells were maintained in Ham's F12 (Irvine Scientific, Irvine, California) supplemented with 5% fetal bovine serum (FBS)(Hyclone Laboratories, Logan, Utah). Cell stocks were stored frozen in liquid nitrogen and an aliquot was thawed as needed for each assay. CHO cells used for the sister chromatid exchange assay were obtained from Dr. S. Wolff, University of California San Francisco, San Francisco, California. These cells were grown in GEM 1717 (Irvine Scientific) supplemented with 10% FBS. No antibiotics were used in either culture system.

Assay Formats

HGPRT forward mutation assay

This assay has 4 stages: dosing, 24-hour cytotoxicity determination, 7-day mutation expression time, and finally quantitation of the mutant frequency in the cultures.

Dosing: Twenty cultures each containing 1.5×10^6 CHO HGPRT $^{+/-}$ cells in Ham's F12 with 5% FBS were exposed to the controls or test compounds for five hours, washed twice, and allowed to continue growing in Ham's F12 with 5% FBS to express whatever toxicity may have been induced by the treatment. Specific doses are given in Table I of the Appendix.

Cytotoxicity: After 24 hours, the cell monolayers were removed from the flasks with Ca^{++}- and Mg^{++}- free Hanks Salt Solution, and single cell suspensions were produced by gentle pipetting. Two hundred cells from each culture were plated into each of three dishes to determine the percentage of viable cells that remained after 24 hours. These plates were incubated for 7 days, fixed, stained with crystal violet, and the colonies counted. Data were expressed in terms of mean raw count (viable count), cloning efficiency (count/200), and percent of negative controls.

Mutation expression: From the remaining cells in each group, 1.5×10^6 cells were put into a fresh flask to continue growing. Since the cells have a doubling time of about 10-12 hours, it is necessary to split the cultures again on day 4 to prevent their overgrowth. Again single

cell suspensions were produced, and 1.5 x 10^6 cells were seeded into fresh flasks.

Quantitation of mutant frequency On day seven, by which time the mutant cells had lost all of their HGPRT enzyme, the populations were ready to undergo selective cloning to determine the relative number of mutant cells induced by each treatment. Both viable count cloning (as on day one) and selective cloning were performed. For the selective cloning of each treatment group, 2 x 10^5 cells were plated into each of 7 dishes which contained selection medium (total of 1.4 x 10^6 cells). Selection medium consisted of Ham's F12 without hypoxanthine but supplemented with 5% dialyzed FBS and 1.34 x 10^{-5} M 6-thioguanine. 6-Thioguanine prevents cells that contain the HGPRT enzyme (nonmutants) from proliferating but does not harm the mutant cells. The viable count cloning was performed, as on day one, using selective medium without 6-thioguanine. After seven days, the plates were fixed, stained, and the colonies counted. Data were expressed as mutants per million viable cells. For example, if the total number of mutant colonies on the seven selective cloning plates were 178 and the viable count were 134, then the mutant frequency would be:

$178/(1.4 \times 10^6) \times (200/134) = 190.7$ mutant clones per 10^6 viable cells

where
$1.4 \times 10^6 = 2 \times 10^5 \times 7$ was the total number of cells plated for the mutant count, and

200/134 was the inverse of the viable fraction and corrected for the percentage of dead cells in the cultures.

In order for a compound to be considered mutagenic it should induce a dose-correlated increase in mutant frequency with at least one dose yielding a threefold rise in the mutant frequency over controls. A single value in the middle of the dose range that produces a threefold increase without the correlated dose response relationship is generally not considered to signify an overall positive response.

CHO sister chromatid exchange assay

The CHO cells were grown and dosed in GEM 1717 supplemented with 10% fetal bovine serum. The sterilized test materials were dissolved directly in the medium. The dose groups and controls are given in Table II, in the Appendix. The cells were exposed for 24 hours to the compound and 5 uM 5-bromodeoxyuridine (BrdU). During the

last 2.5 hours of the growth period, colchicine (0.4 ug/ml final) was added to the medium to arrest mitoses. Cells were then harvested, swollen in hypotonic KCl, fixed, and chromosome spreads prepared. The chromosome preparations were stained using the method of Stetka and Wolff (3) to visualize the sister chromatid exchanges. Scoring was done on coded slides. One hundred cells in each treatment group were scored to determine the percentage of first, second, and third division metaphases. Normally 40 second-division metaphases, which contained all 20 chromosomes, were scored for sister chromatid exchanges (data listed as SCEs per metaphase). Data were analyzed by t-test between the negative and positive controls and ANOVA between the negative control and the test compound groups. The level of significance required for a positive response was $p < 0.001$.

RESULTS

The specific dosing materials, dose concentrations and the resulting mutation and SCE data are given in Tables I and II of the Appendix.

In the CHO HGPRT assay, compounds A and B showed marked toxicity at the highest doses tested (1.4-0.7 mg/ml and 4.0-2.0 mg/ml, respectively). Compound C was only moderately toxic even at the highest dose tested (4.0 mg/ml). As expected, the EMS induced a dose-dependent, statistically significant rise in the mutant frequency. Control frequency was 4.6×10^{-6} while EMS at 0.075 and 0.15 mg/ml induced mutation frequencies of 190.7×10^{-6} and 375.8×10^{-6} respectively. Compound C induced greater than a threefold increase in the mutant frequency (18.3×10^{-6}) at 2.0 mg/ml, but this increase did not correlate with dose. No other significant increases in mutant frequency were observed.

In the CHO sister chromatid exchange assay, compound B was very toxic at doses above 2.0 mg/ml while the other compounds were only slightly toxic. Even at 2.0 mg/ml, cells treated with B showed 29% first division metaphases indicating a slowing of cell cycle times. EMS at 2.4 mg/flask (0.16 mg/ml) induced a statistically significant increase in the number of SCEs (control of 7.3 vs EMS of 13.3). None of the test compounds induced any significant increase in SCEs.

DISCUSSION

In the two assays employed, reactive-polymer and its components showed some cytotoxicity but did not induce significant increases in mutant frequency or SCEs. Both assays were considered valid in that the negative control values were within normal limits and the positive controls

induced statistically significant responses. These data suggest a low genotoxic potential for reactive-polymer. Its cytotoxic action, however, might have an effect on very sensitive tissues (e.g. ocular), and therefore some care in handling this material would be appropriate.

REFERENCES

1. Hsie AW, Casciano DA, Couch DB, Krahn DF, O'Neill JP, Whitfield BL. The use of chinese hamster ovary cells to quantify specific locus mutation and to determine mutagenicity of chemicals. Mut Res 1981; 86:193-214.

2. Latt SA. Sister chromatid exchange, indices of human chromosome damage and repair: Detection by fluorescence induced by Mitomycin C. Proc Natl Acad Sci USA 1974; 71: 3162-3166.

3. Stetka DG, Wolff S. Sister chromatid exchanges as an assay for genetic damage induced. Mutagens/Carcinogens. Part II. In vitro test for compounds requiring metabolic activation. Mut Res 1976: 41: 343-350.

Appendix

Table I

CHO HGPRT FORWARD MUTATION ASSAY

22 OCT 1986

Compound	Dose (mg/ml)	Viable[a] Count (day one)	CE[b]	%C[c]	Viable Count (day seven)	CE	%C	Mutant Frequency (x 10^{-6})
Neg	0.0	171	86	100	174	87	100	4.6
EMS[d]	0.075	134	67	78	133	67	76	190.7[e]
EMS	0.150	97	49	57	92	46	53	375.8[e]
A	1.40	0	0	0	Too toxic			
A	0.70	7	4	4	108	54	62	0.0[f]
A	0.52	111	56	65	152	76	87	7.5[f]
A	0.35	164	82	96	147	74	84	7.8[f]
A	0.26	179	90	105	144	72	83	11.9[f]
A	0.175	175	88	102	143	72	83	2.0[f]
B	4.0	0	0	0	Too toxic			
B	2.0	0	0	0	Too toxic			
B	1.5	8	4	5	121	61	70	0.0[f]
B	1.0	86	43	50	157	79	90	5.4[f]
B	0.75	165	83	96	158	79	91	0.[f]
B	0.5	176	88	103	168	84	97	3.4[f]
C	4.0	114	57	67	160	80	92	5.4[f]
C	2.0	135	67	79	164	82	94	18.3[g]
C	1.5	150	75	88	153	77	88	4.4[f]
C	1.0	136	68	80	158	79	91	5.4[f]

[a] Viable Count= number of colonies formed from 200 cells plated
[b] CE = Cloning Efficiency = viable count/200
[c] %C = percent of controls
[d] EMS= ethyl methanesulfonate
[e] Significant by the threefold-over-controls rule
[f] Not Significant
[g] Meets the threefold-over-controls rule; however, no strong correlated dose response was achieved and therefore M was not considered to be significantly mutagenic.
A= Active B= Reactive-polymer C= Matrix

Table II

SISTER CHROMATID EXCHANGE ASSAY

1 Oct 86

Compound	Dose	%FD	SCE	SD	Comment
Neg Cont.		2	7.1	3.1	
EMS	2.4 mg/flask	2	13.3	4.3	p<0.00001
A	0.75 mg/flask	15	8.0	3.7	NS
A	0.60 mg/flask	2	8.2	2.9	NS
A	0.45 mg/flask	15	7.6	2.8	NS
B	5.0 mg/flask	too toxic to score			
B	4.0 mg/flask	too toxic to score			
B	3.0 mg/flask	too toxic to score			
B	2.0 mg/flask	29	7.8	2.8	NS
B	1.0 mg/flask	12	8.4	2.8	NS
C	3.0 mg/flask	7	6.0	2.8	NS
C	2.0 mg/flask	6	7.5	2.6	NS
C	1.0 mg/flask	7	7.2	3.2	NS
Neg Cont.		6	7.4	2.8	

%FD= percent in first division increase above controls indicates cytotoxicity and slowing of cell cycle.

SCE= sister chromatid exchanges

SD= standard deviation

NS= not significant

Control and treatment groups were compared using ANOVA at $p < 0.001$. Negative control and positive controls were compared using the t-test.

A= Active B= Reactive-polymer C= Matrix

OFFICIAL DISTRIBUTION LIST

Commander
US Army Medical Research
& Development Command
ATTN: SGRD-RMS/Mrs. Madigan
Fort Detrick, MD 21701-5012

Defense Technical Information Center
ATTN: DTIC/DDAB (2 copies)
Cameron Station
Alexandria, VA 22304-6145

Office of Under Secretary of Defense
Research and Engineering
ATTN: R&AT (E&LS), Room 3D129
The Pentagon
Washington, DC 20301-3080

The Surgeon General
ATTN: DASG-TLO
Washington, DC 20310

HQ DA (DASG-ZXA)
WASH DC 20310-2300

Commandant
Academy of Health Sciences
US Army
ATTN: HSHA-CDM
Fort Sam Houston, TX 78234-6100

Uniformed Services University of
Health Sciences
Office of Grants Management
4301 Jones Bridge Road
Bethesda, MD 20814-4799

US Army Research Office
ATTN: Chemical and Biological
Sciences Division
PO Box 12211
Research Triangle Park, NC 27709-2211

Director
ATTN: SGRD-UWZ-L
Walter Reed Army Institute of Research
Washington, DC 20307-5100

Commander
US Army Medical Research Institute
of Infectious Diseases
ATTN: SGRD-ULZ-A
Fort Detrick, MD 21701-5011

Commander
US Army Medical Bioengineering Research
and Development Laboratory
ATTN: SGRD-UBG-M
Fort Detrick, Bldg 568
Frederick, MD 21701-5010

Commander
US Army Medical Bioengineering
Research & Development Laboratory
ATTN: Library
Fort Detrick, Bldg 568
Frederick, MD 21701-5010

Commander
US Army Research Institute
of Environmental Medicine
ATTN: SGRD UE-RSA
Kansas Street
Natick, MA 01760-5007

Commander
US Army Research Institute of
Surgical Research
Fort Sam Houston, TX 78234-6200

Commander
US Army Research Institute of
Chemical Defense
ATTN: SGRD-UV-AJ
Aberdeen Proving Ground, MD 21010-5425

Commander
US Army Aeromedical Research
Laboratory
Fort Rucker, AL 36362-5000

AIR FORCE Office of Scientific
Research (NL)
Building 410, Room A217
Bolling Air Force Base, DC 20332-6448

Commander
USAFSAM/TSZ
Brooks Air Force Base, TX 78235-5000

Head, Biological Sciences Division
OFFICE OF NAVAL RESEARCH
800 North Quincy Street
Arlington, VA 22217-5000